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Physicochemical Properties of Native Adzuki Bean (*Vigna angularis*) 7S Globulin and the Molecular Cloning of Its cDNA Isoforms

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7S globulin (vicilin), the major seed storage protein in adzuki bean [*Vigna angularis*], was purified by ammonium sulfate fractionation, gel filtration column chromatography, and anion-exchange column chromatography that resulted in two fractions. On SDS-PAGE, both fractions gave two major and some minor bands, but there was a difference in the minor band compositions between the two fractions. Thermal stability, solubility, surface hydrophobicity, and emulsifying ability of these three samples were analyzed. Although there was no difference in solubility and emulsifying ability among the samples, thermal stability and surface hydrophobicity were different. These differences might be due to the differences in subunit compositions. cDNAs were cloned by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed on the basis of the determined N-terminal sequences of the major bands. We obtained three isoforms of cDNAs, which had highest homology with the mung bean 8Sα globulin (7S globulin), and then soybean β-conglycinin (7S globulin) β subunit among legume plants. Adzuki bean 7S globulin isoforms contain more methionine and tryptophan than mung bean 8Sα globulin and soybean β-conglycinin β subunit. In addition, high mannose types of glycans were attached to two or one N-glycosylation sites of adzuki bean 7S globulins.

KEYWORDS: Adzuki bean; storage proteins; 7S globulin; physicochemical properties; thermal stability; surface hydrophobicity; *Vigna angularis*

INTRODUCTION

Adzuki bean is a legume that is native to the northeastern part of China. Soybean protein is utilized for various processed foods. On the other hand, although adzuki bean contains proteins which are similar to those of soybean, it is not widely used for processed foods on the basis of the characteristics of proteins. This may be due to the lack of systematic studies on storage protein characteristics unlike those on soybean.

β-Conglycinin (7S globulin) and glycinin (11S globulin) are the major storage proteins in soybean seeds. Both globulins constitute 70−80% of total protein, and thus they determine the characteristics of soybean proteins (*1*). We have investigated the structure−function relationships of soybean proteins (*2*−*11*) and of mung bean 8Sα globulin (7S globulin) in detail (*12*−*15*). Adzuki bean seeds contain mainly 7S globulin as a major storage protein similarly to mung bean. Elucidating and understanding the structure−function relationships of various legume seed storage proteins could lead to their better utilization. Thus, we investigated the structure−function relationships of adzuki bean 7S globulin under the same conditions as those of soybean proteins as a part of our studies aimed at the elucidation of structure−function relationships of various legume seed storage proteins.

The 7S globulin accounts for about 80% of the total proteins of adzuki bean whereas 11S globulin (legumin) makes up only about 10% (*16*). Solubility, emulsification, foaming ability, thermal aggregation, thermal gelation, and flow property of adzuki bean total globulins have been reported (*17*−*20*). However, only the thermal stability of purified 7S globulin has been reported (*16*). To investigate such structure−function relationships, purification of each globulin and analyses of its properties are needed. In this paper, we purified the 7S globulin of adzuki bean and further fractionated it into two fractions with subunit compositions different from each other. We determined and compared their thermal stability, solubility, surface hydrophobicity, and emulsifying ability under the same conditions as those used for soybean protein. We also cloned and sequenced the cDNAs encoding the 7S globulins by reverse transcription-polymerase chain reaction (RT-PCR) using primers designed on the basis of the N-terminal amino acid sequence of the 7S globulins and deduced their amino acid sequences.

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MATERIALS AND METHODS

Plant Materials. Seeds of adzuki bean (*Vigna angularis*) variety Dainagon were purchased from a local seed supplier.

Seeds were sown in pots and developing cotyledons were harvested for the preparation of total RNAs.

Protein Extraction. Seeds were ground using a mill and were defatted with *n*-hexane (1:10 w/v; 1 h; room temperature) and then with acetone (1:10 w/v; 1 h; room temperature). Globulins were extracted from the defatted meal with buffer A (35 mM sodium phosphate, pH 7.6, 0.4 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM (*p*-amidinophenyl) methanesulfonyl fluoride (*p*-APMSF), 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3) at a ratio of 1 g to 10 mL by stirring for 2 h at room temperature. The extract was centrifuged at 9800*g* for 30 min, and the supernatant (crude extract) was collected.

Ammonium Sulfate Fractionation. Solid ammonium sulfate [$(NH_4)_2SO_4$] was slowly added to the crude extract to 60% saturation with stirring on an ice bath. After the sample was kept on ice with stirring for 30 min, it was centrifuged at 4 °C for 30 min (9800*g*). The supernatant was adjusted to 85% saturation with $(NH_4)_2SO_4$, was stirred for 30 min, and was centrifuged again. The precipitate was dissolved in buffer A and was subjected to column chromatography.

Fractionation by Sephacryl S-300 and Mono Q Column Chromatography. Ammonium sulfate-fractionated globulins were applied on a HiPrep 26/60 Sephacryl S-300 HR column (GE Healthcare Bioscience, NJ) at a flow rate of 1 mL/min with buffer A. Fractions were analyzed by SDS-PAGE using 11% polyacrylamide gels according to the procedure of Laemmli (*21*), and those containing mainly the bands belonging to 7S globulin (54−56 kDa) were pooled together as fraction A.

Fraction A was dialyzed against buffer A containing 0.1 M NaCl and was subjected to Mono Q HR 10/10 column (GE Healthcare Bioscience). Proteins were eluted at a flow rate of 2 mL/min with a linear gradient of 0.1−0.4 M NaCl in buffer A.

Protein Measurement. The protein content of samples was determined using a Protein Assay Rapid Kit (Wako Pure Chemical Industries, Japan) with bovine serum albumin as the standard.

Band intensities of fragmented and intact subunits in an SDS-PAGE gel were calculated using the ImageMaster 1D Elite ver. 3.0 densitometric analysis program (GE Healthcare Bio-science).

Differential Scanning Calorimetry (DSC) Measurement. Protein samples were dialyzed against buffer A for high ionic strength (μ = 0.5) and buffer B (10 mM sodium phosphate, pH 7.6, 0.05 M NaCl, 10 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM *p*-APMSF, 1.2 μM leupeptin, 0.2 μM pepstatin A, 0.02% (w/v) NaN_3) for low ionic strength (μ = 0.08). After dialysis, the samples were subjected to the DSC measurement that was carried out on a Microcal MC-2 ultrasensitive microcalorimeter (MicroCal, MA) as described previously (*2*). All DSC experiments were performed with a protein concentration of 0.5 mg/mL. A DSC scan rate of 1 °C/min was used for all experiments.

Solubility as a Function of pH. The solubility of protein samples as a function of pH was measured as described previously (*3*). The protein solutions (0.8 mg/mL) were kept at 4 °C for 18 h at various pHs at μ = 0.5 and μ = 0.08. After centrifugation, protein concentrations in the supernatant were determined using a Protein Assay Rapid Kit (Wako Pure Chemical Industries). Solubility was expressed as percentage of the total protein content in the sample.

Surface Hydrophobicity. Surface hydrophobicities of protein samples were analyzed by two hydrophobic interaction chromatography columns, Phenyl Sepharose 6 Fast Flow and Butyl Sepharose 4 Fast Flow (both from GE Healthcare Bio-science). Samples were dialyzed against buffer A containing 2.3 M ammonium sulfate. The dialyzed samples were applied on columns equilibrated with the same buffer. The adsorbed samples were eluted with a linear gradient (2.3−0 M) of ammonium sulfate at a flow rate of 0.25 mL/min. Elution times of the samples were noted.

Emulsifying Ability. The emulsifying abilities of protein samples were measured as described previously (*3*). Protein samples were dialyzed against buffer A for μ = 0.5 and buffer B for μ = 0.08. Dialyzed samples (1.5 mL at 1.0 mg/mL, μ = 0.5 or 0.08) and 0.25 mL of soybean oil were homogenized and sonicated. Each sample was analyzed three times and the average particle sizes of the emulsions were evaluated.

N-Terminal Amino Acid Sequence Analysis. The main band of fraction A was excised from SDS-PAGE gels, and the proteins were extracted from the gel with SDS buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol), were blotted onto a PVDF membrane by Prosorb cartridge (Applied Biosystems, CA), and were subjected to N-terminal amino acid sequencing using a Procise 492 protein sequencer (Applied Biosystems).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). By using RT-PCR method, we cloned the cDNAs encoding 7S globulin subunits. Total RNA was isolated from developing adzuki bean seeds according to Shirzadegan et al. (*22*). The 7S globulin subunit cDNA was amplified using the RNA LA PCR Kit (AMV), Ver. 1.1 (Takara Bio, Japan). At first, mRNAs in total RNAs were reverse-transcribed by the primer 5′-CGC *GGATCC GGTACC CTGCAG GTCGAC* TTTTTTTTTTTTTTTT-3′ that was composed of the region complementary to poly (A) and four restriction enzyme sites indicated by italics. Then, the product was used for PCR amplification of the cDNA using the primer 5′-ATH GTI CAY MGI GAR CAY CA-3′ corresponding to the N-terminal sequence deduced from N-terminal amino acid sequence of the fraction A. The primer contained mixed bases. At the same time, the primer 5′-CGC *GGATCC GGTACC CTGCAG GTCGAC*-3′ corresponding to the C-terminal four restriction enzyme sites was used. The reaction was performed using LA Taq DNA Polymerase (Takara Bio) with 30 cycles at 94 °C for 30 s, 40 °C for 30 s, and 72 °C for 3 min. The PCR product was further amplified using KOD -Plus- DNA Polymerase (TOYOBO, Japan) with 30 cycles at 94 °C for 15 s, 45 °C for 30 s, and 68 °C for 3 min. The amplified fragment with the expected size was blunted, phosphorylated, and treated with *Bam*HI. Then, the resultant fragment was ligated with pBluescript SK(-) (Stratagene, CA) that was previously treated with *Bam*HI and *Eco*RV and was dephosphorylated. Sequencing was carried out according to the dideoxy method using an ABI Prism 3100 DNA analyzer (Applied Biosystems).

After the sequence was resolved, new primer pairs specific to the 7S globulin coding region were designed. RT reaction was performed as mentioned above, and the primers 5′-ATT GTG CAT CGG GAG CAT C-3′ and 5′-CG*G GAT CC*T TAT **TCA** GTA GAG AC-3′ for DNA regions encoding the N- and C-terminal regions, respectively, were used for the following PCR. The restriction enzyme site (*Bam*HI) and the stop codon are indicated by italics and bold letters, respectively. The reaction was performed using LA Taq DNA Polymerase (Takara Bio) under the same conditions as above. Sufficient quantities of PCR products were obtained, hence, no additional PCR was performed. The subsequent procedure was the same as described above.

Analysis of Carbohydrate Moieties. Glycans were detected by using concanavalin A-horseradish peroxidase (ConA-HRP, Seikagaku Kogyo, Japan) as described previously (*23*, *24*). Purified 7S globulin was separated by SDS-PAGE as mentioned above and was transferred electrophoretically to nitrocellulose membranes. The membranes were soaked in a blocking solution (3% (w/v) skim milk in 10 mM Tris-HCl, pH 7.4, 0.15 M NaCl, 0.05% (v/v) Tween 20) three times each for 10 min. After blocking, the membranes were reacted with ConA-HRP (5 μg/mL) diluted with the blocking solution for 1 h. After rinsing the membranes in a blocking solution two times each for 10 min and in 15 mM sodium phosphate buffer (pH 6.8) two times each for 10 min, proteins on the membranes were reacted with 0.2 mM 3,3′-diaminobenzidine in 0.03% (v/v) hydrogen peroxide.

To clarify numbers and types of carbohydrate moieties, the 7S globulins were denatured in a denaturing buffer (0.5% (w/v) SDS and 40 mM DTT) at 100 °C for 10 min and were cooled to room temperature. The reduced-denatured samples were then treated by Endo H (New England Biolabs, MA) in a reaction buffer (50 mM sodium citrate, pH 5.5, 0.5% (w/v) SDS, 40 mM DTT) at 37 °C for 24 h. The treated samples were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

Isolation and Purification of 7S Globulin from Adzuki Bean. The 60−80% ammonium sulfate fraction of the adzuki

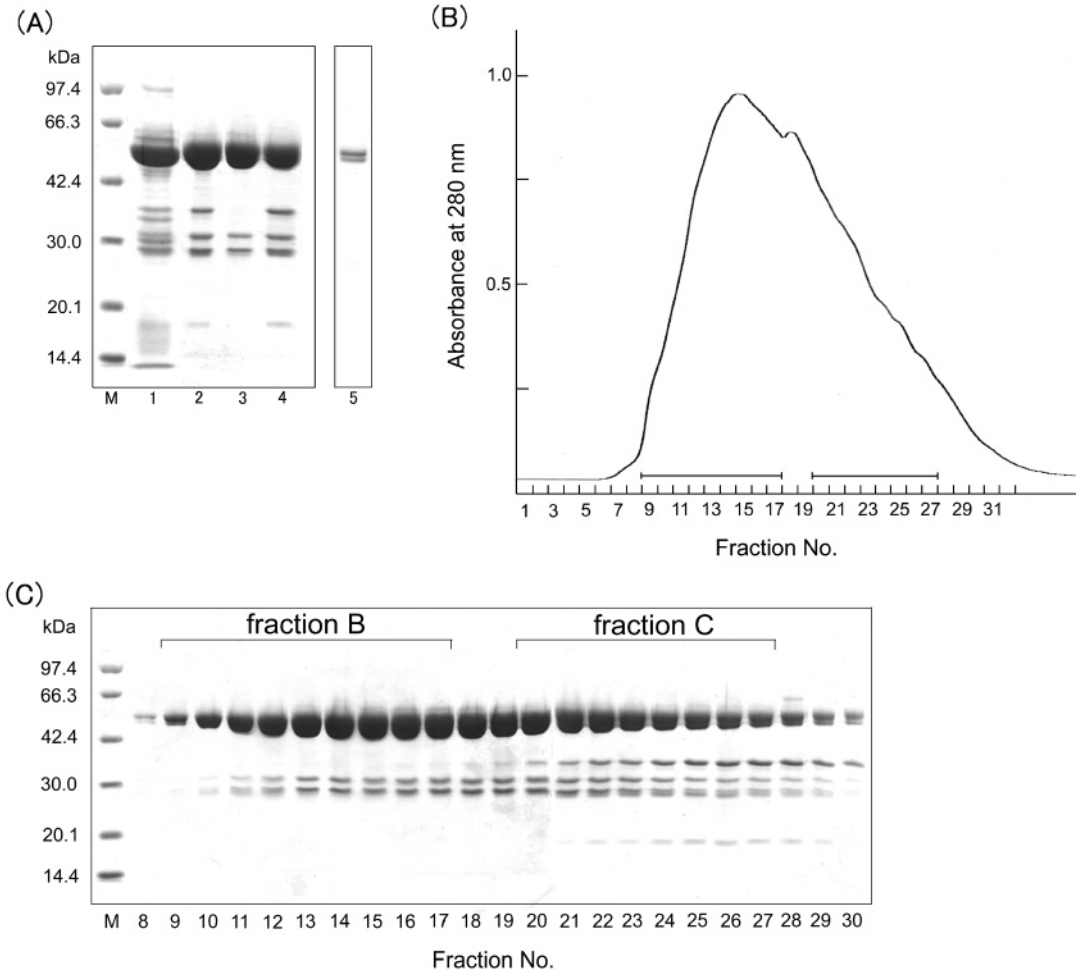

Figure 1. (**A**) SDS-PAGE patterns of collected fractions. M, molecular weight markers; lane 1, adzuki bean extract; lane 2, fraction A; lane 3, fraction B; lane 4, fraction C; lane 5, fraction A of $^1/_{16}$ protein amount compared with that of lane 2. (**B**) Mono Q column chromatography of fraction A. (**C**) SDS-PAGE profiles of eluted fractions. Fractions no. 9–17 and no. 20–27 were collected as fractions B and C, respectively.

bean 7S globulin was applied on the Sephacryl S-300 column, and the major peak was collected as fraction A. Fraction A gave the 54 and 56 kDa major bands and five minor bands of 37, 31, 28, 27, and 17 kDa on SDS-PAGE (**Figure 1A**, lanes 2 and 5). These bands are likely to correspond mainly to 7S globulin according to the results reported by Meng and Ma (*16*). Fraction A was further subjected to Mono Q column chromatography, and a peak with a shoulder was obtained (**Figure 1B**). On SDS-PAGE, fractions corresponding to the major peak exhibited bands similar to those of fraction A, and fractions corresponding to the shoulder had bands close to those of fraction A (**Figure 1C**). We collected the major peak and the shoulder separately as fractions B and C, respectively (**Figure 1A**, lanes 3 and 4). Considering the size of the major band, the protein seems to be a type of the soybean β-conglycinin β subunit, which lacks the extension region and not α and α' subunits which have the extension region (*2*). The physicochemical properties of the adzuki bean 7S globulin will be subsequently compared with those of the soybean β-conglycinin β subunit homotrimer (β homotrimer).

Thermal Stability. The structural stability of proteins is an important factor for their gel-forming and emulsifying abilities. Thermal partial denaturation is a prerequisite for the formation of the gel network structure.

DSC profiles of fractions A, B, and C at $\mu = 0.5$ are shown in **Figure 2A**. Previously, our group reported that the β homotrimer had a single peak with the thermal denaturation midpoint temperatures (T_m) of 87.0 °C at $\mu = 0.5$ (*6*). Our results show that fraction A exhibited a major and a minor peak at 85.4 °C and 92.3 °C; fraction B, 85.5 °C and 92.4 °C; and fraction C, 92.1 °C and 84.9 °C, respectively. Meng and Ma also observed two peaks and speculated that the higher T_m value corresponds to glycosylated subunits of 7S globulin (*16*). However, when we prepared recombinant proteins using *Escherichia coli* expression system and measured their T_m values at $\mu = 0.5$, they gave T_m values corresponding to the higher T_m values of the native 7S globulin (will be described elsewhere). Since no glycans are attached to proteins expressed in *E. coli*, the results indicate that the higher T_m value of fractions A, B, and C is not due to the glycosylated 7S globulin subunits. This is in accordance with our previous report that glycans do not contribute to the thermal stability of soybean β-conglycinin (*2*, *6*).

As there is a quite big difference in the amount of 37−17 kDa fragments between fractions B and C, the difference in the subunit composition might influence the thermal stability. Therefore, the intensities of the bands in **Figure 1A**, lanes 3 and 4, were calculated. As shown in **Figure 3**, the ratios of fragmented subunits (37, 31, 28, 27, and 17 kDa) to intact subunits (56 and 54 kDa) are 7.7:92.3 and 23.5:76.5 in fractions B and C, respectively. The ratios of trimers containing one or no fragmented subunit should be 23.1:76.9 (1:3.3) and 70.5:29.5 (2.4:1), respectively. These ratios roughly correspond to the peak ratios of fractions B and C, respectively, suggesting

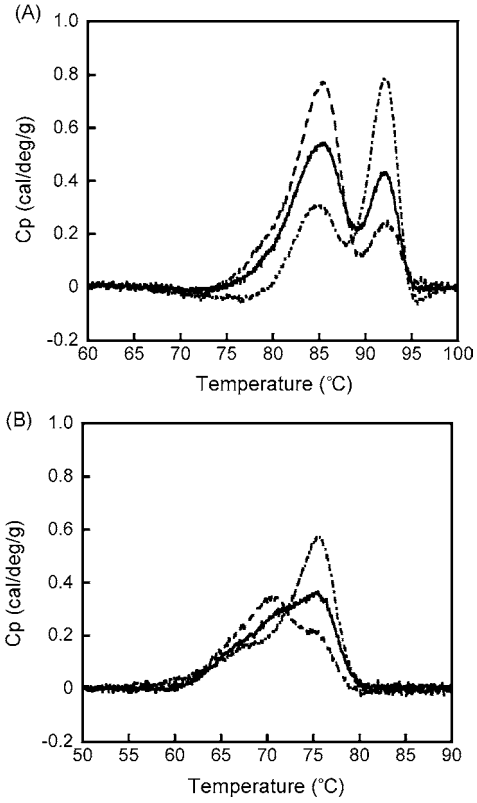

Figure 2. DSC scans of fractions A, B, and C at $\mu = 0.5$ (**A**) and 0.08 (**B**). Profiles of fractions A, B, and C are shown by solid line, dashed line, and dashed and single-dotted line, respectively.

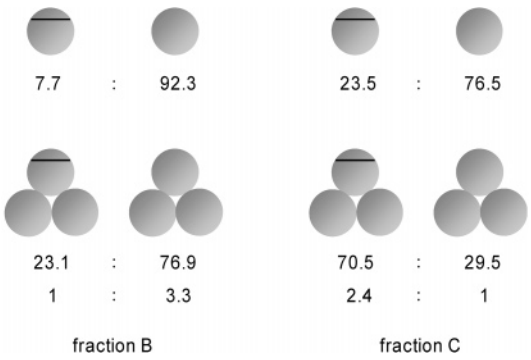

Figure 3. Ratios of monomers and trimers containing one or no fragment in fractions B and C. Spheres with and without bars correspond to fragmented subunits and intact subunits, respectively.

that inclusion of a fragmented subunit in a trimer stabilizes the trimer in spite of fragmentation patterns revealed by SDS-PAGE (**Figure 1A**). Usually, fragmentation of a protein results in destabilization (*25*). So, this finding is the common least example.

At $\mu = 0.08$, fractions A, B, and C gave different patterns from those at $\mu = 0.5$; T_m values were lower (**Figure 2B**). Fraction A exhibited a broad peak at 75.7 °C with a shoulder at 70.5 °C. On the other hand, fractions B and C had peaks at 70.5 °C and 75.7 °C with a shoulder of 75.6 °C and 67.1 °C, respectively.

Solubility as a Function of pH. Generally, it is said that the higher the solubility of proteins, the higher its gel-forming, emulsifying, and foaming abilities (*26*). We compared the solubilities of fractions A, B, and C with that of the β homotrimer at $\mu = 0.5$ and 0.08 (**Figure 4**). At $\mu = 0.5$, all fractions A, B, and C were soluble similar to the β homotrimer

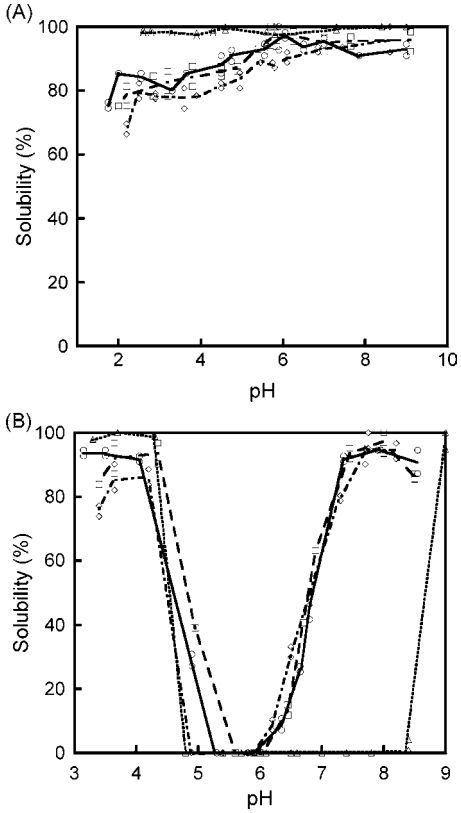

Figure 4. pH dependence of solubilities of fractions A, B, and C and β homotrimer at $\mu = 0.5$ (**A**) and 0.08 (**B**). Fractions A, B, and C and β homotrimer are shown by solid line with circles, dashed line with squares, dashed and single-dotted line with diamonds, and dotted line with triangles, respectively.

Table 1. Elution Times of Each Fraction on Hydrophobic Columns

	elution times (min)			
column	fraction A	fraction B	fraction C	β homotrimer
butyl sepharose	37.0	37.2	38.0	38.2
phenyl sepharose	61.4	57.9	64.5	56.7

at pH around 6−9 but exhibited slightly lower solubilities at pH < 6. At $\mu = 0.08$, fractions A, B, and C exhibited isoelectric precipitation at pH around 5−6; in contrast, the β homotrimer precipitated at a wide range of pH 4.8−8.5.

The difference in the solubilities between the α and α′ homotrimers and the β homotrimer is due to the presence of the extension regions in the α and α′ homotrimers (*2*). However, in spite of lacking the extension regions, adzuki bean 7S globulin exhibited better solubilities than those of the β homotrimer at $\mu = 0.08$. This result suggests that there may be differences in the molecular surfaces between adzuki bean 7S globulin and β homotrimer. It is reported that the factor determining solubility is not the amino acid composition but the differences in the distribution and the localization of hydrophobic and charged residues on molecular surface (*10*). Thus, surface hydrophobicity was analyzed.

Surface Hydrophobicity. We assessed the surface hydrophobicities of the fractions A, B, and C and the β homotrimer by measuring their elution times on a hydrophobic column (i.e., the longer the elution time, the higher the surface hydrophobicity). We used two columns containing a phenyl and a butyl group (**Table 1**). The four samples had similar elution times on the butyl sepharose column. On the other hand, fraction C

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Adzuki 7S1    1  IVHREHHESREEVSVSSGKNNPFYFNSDRWFRTLYRNEWGHIRVLQRFDQRSKQMQNLENYRVVEFKSKPNTLLLPHHADADFLLVVLNGTAVLTLVNPD  100
Adzuki 7S2    1  IVHREHHESREEVSVSSGKNNPFYFNSDRWFRTLYRNEWGHIRILHRFDQRSKQIQNLENYRVVEFKSKPNTLLLPHHADADFLLVVLNGRAILTLVNPD  100
Adzuki 7S3    1  IVHREHQESQEEVSDSRGENNPFYFSSDRRFHTLFTNQYGHIHILHRFDQRSKQIQNLENYRVVEFKSKPNTLLLPHHADADFLLVVLNGRAILTLVNPD   99
Soybean β     1  ----------LKVREDENNPFYFRSSNSFQTLFENQNGRIRLLQRFNKRSPQLENLRDYRIVQFQSKPNTILLPHHADADFLLFVLSGRAILTLVNND   88

Adzuki 7S1  101  SRDSYILEQGHAQKIPAGTTFFLVNPDDNENLRIIKLAIPVNNPHRFQDFFLSSTEAQQSYLRGFSKNILEASFDSDFKEINRVLFGEERQQQQGEESRE  200
Adzuki 7S2  101  SRDSYILEQGHAQKIPAGTTFFLVNPDDNENLRIIKLAIPVNNPHRFQDFFLSSTEAQQSYLRGFSKNILEASFDSDFKEINRVLFGEERQQQQGEESRE  200
Adzuki 7S3  100  SRDSYILEQGHAQKIPAGTTFFLVNPDDNENLRIIKLAIPVNNPHRFQDFFLSSTEAQQSYLRGFSKNILEASFDSDFKEINRVLFGEERQQQQGEESRE  199
Soybean β    89  DRDSYNLHPGDAQRIPAGTTYYLVNPHDHQNLKIIKLAIPVNKPGRYDDFFLSSTQAQQSYLQGFSHNILETSFHSEFEEINRVLFGEEEEQRQ-----Q  183

Adzuki 7S1  201  EGVIVELKREQIQELMKHAKSSSRKELSSQDEPFNLRNSKPIYSNKFGRWYEMTPEKNPQLKDLDVFISSVDMKEGALLLPHYSSKAIVIMVINEGEAKI  300
Adzuki 7S2  201  EGVIVELKREQIQELMKHAKSSSRKELSSQDEPFNLRNSKPIYSNKFGRWYEMTPEKNPQLKDLDVFISSVDMKEGALLLPHYNSKAIVIMVINEGEAKI  300
Adzuki 7S3  200  EGVIVELKREQIQELMKHAKSSSRKELSSQDEPFNLRNSKPIYSNKFGRWYEMTPEKNPQLKDLDVFISSVDMKEGALLLPHYNSKAIVIMVINEGEAKI  299
Soybean β   184  EGVIVELSKEQIRQLSRRAKSSSRKTISSEDEPFNLRSRNPIYSNNFGKFFEITPEKNPQLRDLDIFLSSVDINEGALLLPHFNSKAIVILVINEGDANI  283

Adzuki 7S1  301  ELVGLSDQQQ-QKQQEESLEVQRYRAELSEDDVFVIPAAYPVAINATSNLNFFAFGINAENNRRNFLAGGKDNVMSEIPTEVLEVSFPASGKKVEKLIKK  399
Adzuki 7S2  301  ELVGLSDQQQ-QKQQEESLEVQRYRAELSEDDVFVIPAAYPVAINATSNLNFFAFGINAENNQRNFLAGGKDNVMSEIPTEVLEVSFPASGKKVEKLIKK  399
Adzuki 7S3  300  ELVGLSDQQQ-QKQQEESLEVQRYRAELSEDDVFVIPAAYPVAINATSNLNFFAFGINAENNQRNFLAGGKDNVMSEIPTEVLEVSFPASGKKVEKLIKK  398
Soybean β   284  ELVGIKEQQQKQKQEEEPLEVQRYRAELSEDDVFVIPAAYPFVVNATSNLNFLAFGINAENNQRNFLAGEKDNVVRQIERQVQELAFPGSAQDVERLLKK  383

Adzuki 7S1  400  QSESHFVDAQPEQQQREEGHKGRKGSLSSILGSLY  434
Adzuki 7S2  400  QSESHFVDAQPEQQQREEGHKGRKGSLSSILGSLY  434
Adzuki 7S3  399  QSESHFVDAQPEQQQREEGHKGRKGSLSSILGSLY  433
Soybean β   384  QRESYFVDAQP--QQKEEGSKGRKGPFPSILGALY  416
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Figure 5. Alignment of the amino acid sequences of adzuki 7S1, adzuki 7S2, adzuki 7S3, and soybean β subunit. The regions marked by the rectangles and an ellipse indicate amino acid substitutions and a deletion, respectively. N-glycosylation sites are expressed by asterisks. The nucleotide sequences reported in this paper have been deposited to the DDBJ/EMBL/GenBank with accession numbers AB292246, AB292247, and AB292248.

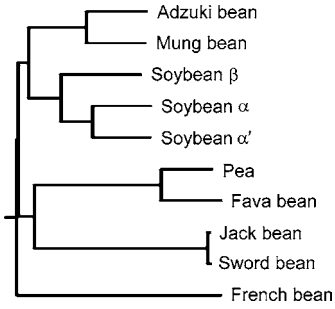

Figure 6. Homology analysis of the amino acid sequences of adzuki bean 7S globulin isoforms and other legume 7S globulins as shown by a dendrogram (ClustalW) (http://align.genome.jp/). The branch length corresponds to the genetic distance among proteins. DDBJ/EMBL/GenBank accession numbers are as follows: soybean β, β-conglycinin β (AB008679); soybean α, β-conglycinin α (AB008678); soybean α', β-conglycinin α' (AB008680); mung bean, 8Sα (DQ538333); pea (Y00722); fava bean (Y00462); jack bean (X59467); sword bean (X06733); French bean (A11822).

had higher hydrophobicity than fraction B and β homotrimer as evaluated by the phenyl sepharose column, although that of fraction B and the β homotrimer had similar levels. The hydrophobicity of fraction A was an arithmetic average of fractions B and C. This means that fraction C has more aromatic amino acids on the surface than fraction B and the β homotrimer, while they had similar number of aliphatic amino acids. However, there is no distinct difference in the solubilities of fractions B and C. Therefore, these results do not provide sufficient information about the differences in the solubility between adzuki bean fractions and β homotrimer. It is necessary to elucidate the adzuki bean 7S globulin structure and to compare the distribution of the electrostatic potential with that of the β homotrimer as has been done for mung bean 7S globulin and soybean 7S globulin β subunit (*15*).

Table 2. N-Terminal Amino Acid Sequences of Minor Bands That Are Assumed to Be Other Types of 7S Globulins

samples	N-terminal sequences
adzuki 7S1	I V H R E H H E S R
adzuki 7S2	I V H R E H H E S R
adzuki 7S3	I V H R E H Q E S Q
37 kDa of fractions A and C	I V H V E E R Q
28 kDa of fractions A and C	I V H R E E H
28 kDa of fraction B	I V E A/Y K P F D L E
27 kDa of fractions A and C	I V H R E E H
31 kDa of fractions A, B, and C	S K K A L S S
17 kDa of fractions A and C	K L Q E E Q Q

Emulsifying Ability. The emulsifying ability of proteins is one of the useful physicochemical properties for food processing. We assessed the emulsifying abilities of samples by measuring the size of the particles after emulsification at $\mu = 0.5$ and 0.08 by homogenization and sonication. The smaller the particle sizes are, the better the emulsifying ability is.

At $\mu = 0.5$ and pH 7.6, the average particle sizes of the emulsions of fractions A, B, and C were similar to that of the β homotrimer and larger than those of the α and α' homotrimers (data not shown). Differences in size among the fractions were not detected. At $\mu = 0.08$ and pH 7.6, the β homotrimer was not measurable because of its insolubility. Although fractions A, B, and C were soluble similar to the α and α' homotrimers under this condition, the particle sizes were larger than those of the α and α' homotrimers (*6*). This confirms the previous observation that the extension regions of α and α' homotrimers contribute to emulsifying abilities.

Surface hydrophobicity has been reported to be an important factor for physicochemical functions such as the emulsifying

Table 3. Amino Acid Compositions of Legume Seed 7S Globulins

	count											
	adzuki bean			soybean								
amino acid	7S1	7S2	7S3	β	α	α'	mung bean 8Sα	pea	fava bean	jack bean	sword bean	French bean
Ala	22	22	22	22	23	22	22	18	16	23	23	21
Arg	26	26	25	29	43	36	24	26	24	26	26	17
Asn	29	30	29	33	36	34	31	35	36	29	31	30
Asp	20	20	21	21	27	26	21	24	20	23	23	21
Cys	0	0	0	0	1	1	0	0	0	2	2	0
Gln	29	29	32	33	46	51	36	34	29	26	26	23
Glu	43	43	43	37	76	79	39	45	45	36	36	36
Gly	21	21	21	19	24	28	22	22	26	24	24	22
His	12	13	13	8	6	20	8	8	9	10	9	10
Ile	23	26	25	26	30	27	24	24	27	21	20	24
Leu	39	39	40	42	45	40	37	43	46	51	52	38
Lys	28	28	27	21	32	39	27	30	32	20	19	24
Met	6	5	5	0	1	2	4	0	0	4	4	3
Phe	25	25	26	28	27	27	27	22	22	19	19	23
Pro	18	18	18	21	38	33	19	18	20	20	20	14
Ser	40	39	39	31	39	40	31	36	35	34	34	36
Thr	10	9	10	10	11	12	12	12	11	15	15	14
Trp	3	3	1	0	1	2	3	0	0	0	0	1
Tyr	11	11	11	12	13	13	8	9	13	15	15	13
Val	29	27	25	23	24	27	28	25	25	21	21	27
total	434	434	433	416	543	559	423	431	436	419	419	397

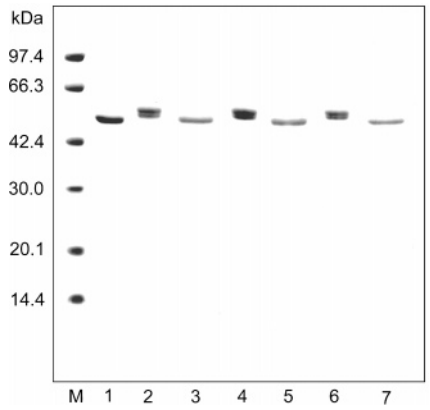

Figure 7. Effect of Endo H treatment on the mobilities of fractions A, B, and C on SDS-PAGE. M, molecular weight markers; lane 1, recombinant adzuki 7S1; lanes 2 and 3, fraction A; lanes 4 and 5, fraction B; lanes 6 and 7, fraction C. Lanes 1, 2, 4, and 6 are without and lanes 3, 5, and 7 are with Endo H treatment.

ability, because it is related to the binding of a protein to oil (*27*, *28*). In addition, a suitable conformational change at the interface between oil and water, structural flexibility, also contributes to physicochemical function (*29*). Generally, a balance between hydrophobicity and hydrophilicity of a protein is a factor related to its emulsifying ability (*29*). Moreover, we previously demonstrated that the extension region and the structural stability are important factors for the emulsifying abilities of soybean 7S globulin and that the carbohydrate moieties do not have influence on them (*3*, *6*, *7*). The surface hydrophobicities of fraction B and fractions A, B, and C assessed by phenyl and butyl sepharose columns, respectively, were similar to that of β homotrimer. However, the hydrophobicity of fraction C assessed by phenyl sepharose column was higher than those of fraction B and β homotrimer. Although all fractions A, B, and C gave two endothermic peaks at $\mu = 0.5$, the order of their overall thermal stability is fractions C > A > B on the basis of the ratio of the two peaks (see **Figure 2A**). This order is also true for the results at $\mu = 0.08$. As high thermal stability is probably related to low structural flexibility, suitable conformational change at the interface between oil and water must not occur. Therefore, the high thermal stability of fraction C brings about a negative effect on its emulsifying ability. Taking into account the observations that both the thermal stability at $\mu = 0.08$ and the surface hydrophobicity of fraction B were similar to those of β homotrimer, that the thermal stability and the surface hydrophobicity of fraction C were higher than those of the others, and that both adzuki bean 7S globulin and soybean β subunit do not have extension regions, we can explain why the emulsifying ability and stability of adzuki bean 7S globulin are similar to those of β homotrimer. The results indicate that the balance of hydrophobic and hydrophilic residues on adzuki bean 7S globulin molecular surfaces is not suitable for emulsification. This will be better explained after elucidation of three-dimensional structures of the adzuki bean 7S globulin.

Cloning of the Adzuki Bean 7S globulin cDNAs. The N-terminal nine amino acid sequence of the adzuki bean 7S globulin major band was I V H R E H H/Q E S. We, therefore, used oligonucleotides synthesized on the basis of the nucleotide sequences corresponding to I V H R E H H/Q as a primer for RT-PCR cloning. As the first and second nucleotides of the codon assigned to the seventh His and Gln are the same, we adopted up to the second nucleotide of the seventh codon.

We cloned one RT-PCR product and sequenced and designed new primers specific to the determined nucleotide sequences corresponding to N- and C-terminal regions. By RT-PCR cloning using these new primers, two kinds of nucleotide sequences were additionally obtained. The deduced amino acid sequences of the adzuki bean 7S globulin isoforms are shown with that of the soybean β subunit in **Figure 5**. Adzuki 7S1 was derived from the first RT-PCR product, and adzuki 7S2 and adzuki 7S3 are from the products obtained by using the new primers. Adzuki 7S2 was found to have 7 amino acid substitutions while adzuki 7S3 has 20 amino acids substitutions and one deletion compared with adzuki 7S1, respectively. Considering that adzuki 7S1 was the product obtained by using the mixed primer, adzuki 7S1 may be the major type of 7S

globulin in adzuki bean seeds, and adzuki 7S2 and adzuki 7S3 may be the second major 7S globulins.

On the basis of their amino acid sequences, a homology analysis of the adzuki bean 7S globulin isoforms and various legume 7S globulins shows highest identity of the adzuki bean 7S globulin with the mung bean 8Sα globulin and then with the soybean β-conglycinin β subunit (**Figure 6**). The amino acid sequence of adzuki 7S1 was adopted as that of the adzuki bean 7S globulin. The sequence identities between adzuki 7S1, adzuki 7S2, and adzuki 7S3 and the mung bean 8Sα globulin are 83%, 83%, and 82%, respectively. In the case of the β-conglycinin β subunit, they are 67%, 68%, and 69%, respectively.

As shown in **Table 2**, when we analyzed the N-terminal sequences of the 37, 31, 28, 27, and 17 kDa bands of fractions A, B, and C, we observed five kinds of sequences. Three of them (37, 28, and 27 kDa of fractions A and C) were very similar to those of adzuki 7S1, adzuki 7S2, and adzuki 7S3. The 28 kDa of fraction B was quite different from the others, although the first two amino acids were Ile-Val, which is a common feature of the major adzuki bean 7S globulin bands. On the other hand, the 31 and 17 kDa bands had N-terminal sequences completely different from any sequences in the 7S globulin isoforms. These fragments may be derived from limited proteolysis of three minor adzuki bean 7S globulins, which have different sequences from the major adzuki bean 7S globulin bands.

Amino Acid Compositions. We compared the amino acid compositions of adzuki 7S1, adzuki 7S2, and adzuki 7S3 with those of the other 7S globulins of legume seeds (**Table 3**). Adzuki 7S1 and adzuki 7S2 contain three tryptophan and 5−6 sulfur-containing amino acids, but the contents of these amino acids in the other 7S globulins are less than those of adzuki 7S1 and adzuki 7S2. This means that the adzuki bean 7S globulin is the richest in essential amino acids among these 7S globulins.

Analysis of Carbohydrate Moieties. We confirmed that fractions A, B, and C have carbohydrate moieties by using the ConA-HRP method (data not shown). The deduced amino acid sequences of adzuki 7S1, adzuki 7S2, and adzuki 7S3 indicate that adzuki 7S1 has two potential N-glycosylation sites at N89 and N344 and that adzuki 7S2 and adzuki 7S3 have only a single site at N344 and N343, respectively (**Figure 5**). To investigate the number of glycans and whether these are high mannose types or not, we treated adzuki bean 7S globulin fractions A, B, and C by Endo H. Endo H removes high mannose types of N-linked carbohydrates. As shown in **Figure 7**, Endo H-treatment of all samples gave a single band with the same size as that of the recombinant one (this will be described elsewhere), which is not glycosylated. This indicates that all carbohydrate moieties at the potential sites are high mannose types and that the upper band of undigested samples corresponds to adzuki 7S1 which has two N-glycosylation sites and the lower band corresponds to adzuki 7S2 and adzuki 7S3 which have one site. As the intensity of the upper band was stronger than that of the lower one, we can say that the upper band is the major 7S globulin, as discussed above.

The adzuki bean 7S globulins were rich in sulfur-containing amino acids and tryptophan among 7S globulins from many legume seeds. This is an impact for food usage. The adzuki bean 7S globulin subunits were shown to have different physicochemical properties from each other. Adzuki 7S1, adzuki 7S2, and adzuki 7S3 are now under crystallization. The structure-physicochemical function relationships of these 7S globulins will be clarified after completion of their X-ray crystallography.

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